Compte rendu de mission à l'ICGEB

(Italie)

P.-Y. Teycheney

3-15 juin 2007

1- Présentation générale de la mission

L'équipe de virologie du CIRAD Guadeloupe collabore depuis 2006 avec l'équipe de Mark Tepfer, DR2 INRA détaché à l'ICGEB (*International Centre for Genetic Engineering and Biosafety*, Ca'Tron du Roncade, Italie) sur la thématique pararetrovirus endogènes. Cette collaboration s'est notamment concrétisée en 2007 par le dépôt auprès de l'ANR du projet *Innovir* (« innovative antiviral strategies ») associant également l'équipe de Mireille Jacquemond (INRA Montfavet).

Dans le cadre de cette collaboration, M. Tepfer et moi-même souhaitions organiser un séminaire international sur les séquences virales intégrées dans le génome des plantes. La présence de ces séquences résulte soit d'une intégration artificielle par génie génétique, à des fins de protection, soit d'évènements de recombinaison illégitime intervenus lors de la réplication de virus au cours de processus naturels d'infection. Ces deux types de séquence présentent, dans un certain nombre de cas, des points communs au niveau de l'induction de mécanismes de résistance antivirale et du risque d'émergence de nouvelles espèces virales. Le rassemblement de chercheurs travaillant sur ces deux types de séquence et/ou sur des thématiques proches (éléments transposables, recombinaison, retrovirus endogènes humains, polydnavirus et évolution) avait pour objectif de faire le point sur les recherches engagées dans ces domaines et de dessiner les axes de possibles collaborations dans le cadre des prochains appels à projet européens du 7^{e} programme cadre.

Pour cela, une demande de financement a été faite en ce sens auprès de la Commission Européenne dans le cadre d'une SSA (projet *Biosafenet*, coordonné par J. Schiemann). Une demande de financement d'action incitative a également été déposée auprès de la Direction Scientifique du CIRAD début 2007. Le projet *Biosafenet* ayant été accepté pour financement par la Commission fin 2006, et la demande de financement d'action incitative par le CIRAD en 2007, il a été décidé d'organiser la manifestation du 6 au 8 juin 2007 sur le campus de la Fondazione Cassamarca situé à Ca'Tron di Roncade.

Par ailleurs, un autre objectif de ma mission était de poursuivre les expérimentations communes débutées avec l'équipe de M. Tepfer en 2006. Celles-ci ont pour but de mettre au point des pararetrovirus endogènes expérimentaux dans des espèces hôtes pour lesquelles existent des outils de génomique puissants (banques EST, séquence du génome, banques de mutants), afin de caractériser les gènes de plantes impliqués dans l'activation des EPRV pathogènes.

2- Premier séminaire *Biosafenet*

Le séminaire a réuni vingt sept participants, dont vingt et un invités, provenant de 10 pays européens (Allemagne, Autriche, Espagne, France, Grèce, Hongrie, Italie, Pays-Bas, Royaume-Uni, Suisse) et d'Israël. La liste complète des participants, le programme et les résumés des communications sont fournis en annexe de ce document.

Cinq sessions avaient été organisées. Elles portaient respectivement sur les thématiques suivantes :

- Plantes transgéniques exprimant des séquences virales et mécanismes des résistances antivirales
- Biosécurité des plantes transgéniques exprimant des séquences virales
- Evolution des populations virales et maladies virales émergentes

- Séquences virales endogènes des plantes
- Séquences virales endogènes des mammifères et des insectes

Les communications, de très grande qualité, et les discussions qui les ont suivi ont permis de faire un point complet sur l'état d'avancement des recherches en Europe (et Israël) sur les séquences virales intégrées dans le génome des plantes. Les communications présentées sur des thématiques proches ont été particulièrement éclairantes. C'est notamment le cas de celle portant sur les bracovirus, une famille de virus endogènes des guêpes indispensables au parasitisme par ces dernières de leurs hôtes naturels lépidoptères (JM Drezen, Université de Tours). De même, les deux communications portant sur l'évolution des populations virales (S. Elena Fito, CSIC Valence et R. Froissart, CNRS Montpellier) ont apporté un éclairage intéressant sur les risques d'émergence et de sélection de nouvelles espèces virales résultant d'évènements de recombinaison entre séquences virales intégrées (ou leurs transcrits) et génomes viraux. Enfin, la communication de B. Hohn (FMI, Bâle) sur la recombinaison et la mémoire trans-générationnelle chez les plantes a permis de replacer la thématique du séminaire dans une perspective plus large. Le séminaire s'est conclu par une table ronde visant à identifier des thèmes fédérateurs susceptibles de générer des projets de recherche communs.

Globalement, cette manifestation a confirmé l'existence de fortes synergies et de problématiques communes entre les équipes travaillant sur les deux types de séquences virales intégrées, notamment en ce qui concerne les mécanismes antiviraux induits par ces séquences et leur contribution à l'émergence de nouvelles populations virales. Ces thématiques présentent également un intérêt pour les équipes travaillant sur les séquences virales endogènes animales et/ou d'insectes. Elles ont donc vocation à générer des projets très transversaux associant les domaines animaux et végétaux. La constitution d'un réseau, amorcée lors de ce séminaire et résultant de projets européens du 5^{e} PCRD (Paradigm, VRTP Impact), devrait favoriser l'émergence de projets communs et/ou de réponses communes à des appels à projet, notamment européens. D'un point de vue institutionnel, la présentation de ML Caruana a permis d'accroître la visibilité du CIRAD sur la thématique pararetrovirus endogènes (EPRV).

Ce séminaire fera l'objet d'un compte rendu destiné à la Commission Européenne et d'un article de synthèse rédigé par M. Tepfer et moi-même, que nous souhaitons publier dans une revue à facteur d'impact de rang A.

3- Collaboration scientifique avec l'ICGEB

Une collaboration scientifique avec l'ICGEB a été entamée en 2006 sur la thématique des EPRV. M. Tepfer étudie depuis de nombreuses années la biosécurité des plantes transgéniques exprimant des séquences virales (*virus resistant transgenic plants*, VRTP), avec un intérêt particulier pour le risque d'émergence de populations virales résultant d'évènements de recombinaison entre transcrits de transgènes viraux et génomes viraux ARN. Or, les plantes hébergeant des EPRV constituent des modèles de plantes transgéniques naturelles particulièrement adaptés à l'évaluation des risques d'émergence de nouvelles populations virales (à génome ADN). Cependant, la collaboration établie avec l'équipe de M. Tepfer porte plus spécifiquement sur l'obtention d'EPRV expérimentaux, à des fins de caractérisation des gènes de plantes impliqués dans l'activation des EPRV pathogènes.

Pour cela, des lignées de *N. tabacum* (cv XHFD8) et *N. benthamiana* transformées transgéniques ont été créées. Une construction fournie par M. Jacquemond (INRA Montfavet) et contenant 1.5 copies du génome du pararetrovirus du gonflement des tiges du cacaoyer (*Cocoa swollen shoot virus*, CSSV) placé sous la dépendance du promoteur 35S du virus de la mosaïque du chou fleur (*Cauliflower mosaic virus*, CaMV) et clonée dans le vecteur binaire pBIN19 a été utilisée pour leur transformation. La caractérisation par PCR et par southern de la première génération d'autofécondation (F1) montre que la plupart des plantes régénérées sur milieu sélectif contenant de la kanamycine sont transformées de façon stable et comportent une copie unique du transgène. Des essais d'activation de la séquence virale introduite seront prochainement effectués, avec pour contrôles positifs des plants de cacao infectés lors de ma mission par bombardement de graines de cacaoyers par le clone infectieux de CSSV.

Par ailleurs, durant mon séjour à l'ICGEB, j'ai débuté une série d'expérimentations visant à exprimer des EPRV BSV infectieux en système hétérologue (tabac, arabette). Ces travaux ont également pour objectifs de caractériser les gènes de plante impliqués dans l'expression des EPRV pathogènes, à des fins de mise au point de stratégies de lutte contre les EPRV, et plus particulièrement contre les EPRV BSV.

Tous ces travaux s'inscrivent dans le cadre d'une collaboration tripartite CIRAD/ICGEB /INRA formalisée en mars 2007 par le dépôt d'un projet commun, dans le cadre de l'appel ANR Blanc.

Annexe 1 : liste des participants au premier séminiare Biosafenet

Ervin Balazs (Agricultural Research Institute, Hongrie)
Marie-Line Caruana (CIRAD Montpellier, France)
Rémi Charrel (University of Marseilles, France)
Laura Chiappetta (ICGEB, Biosafety Outstation, Ca' Tron, Italie)
Wendy Craig (ICGEB, Trieste, Italie)
Marie Dewannieux (University College, London, Royaume-Uni)
Jean Michel Drezen (Université de Tours, France)
Santiago Elena (IBMCP, Valencia, Espagne)
Arianna Friscina (ICGEB, Biosafety Outstation, Ca' Tron, Italie)
Rémy Froissart (CNRS Montpellier, France)
Barbara Hohn (Friedrich Miescher Institut, Basel, Suisse)
Thomas Hohn (Basel University, Suisse)
Mireille Jacquemond (INRA Montfavet, France)
Edgar Maiss (University of Hannover, Hannover, Allemagne)
Klaus Minol (Genius, Darmstadt, Allemagne)
Marco Morroni (ICGEB, Biosafety Outstation, Ca' Tron, Italie)
Marcel Prins (Keygene, Wageningen, Pays-Bas)
Katja Richert-Pöggeler (IPK, Gatersleben, Allemagne)
George Sakellaris (Institute of Biological Research, Athens, Grèce)
Joachim Schiemann (Federal Biological Reseach Centre, Braunschweig, Allemagne)
Trude Schwartzacher (University of Leicester, Royaume-Uni)
Ilan Sela (Hebrew University of Jerusalem, Israël)
Christina Staginnus (Gregor Mendel Institut, Vienna, Autriche)
Mark Tepfer (ICGEB, Ca'Tron, Italie)
Pierre-Yves Teycheney (CIRAD Guadeloupe, France)
Jeremy Thompson (ICGEB, Biosafety Outstation, Ca' Tron, Italie)
Ralph Wilhelm (Federal Biological Research Centre, Braunschweig, Allemagne)

Annexe 2 : Annexe : liste des orateurs invités, programme et résumé des communications du premier séminaire Biosafenet

The aim of the BIOSAFENET project is to facilitate communication about scientific issues related to GMO biosafety

The first BIOSAFENET Seminar:

Balancing resistance and risk: plant endogenous viral sequences and virus-resistant transgenic plants as possible sources of resistance and virus emergence

6-8 June 2007

Invited speakers

Marie-Line Caruana (CIRAD, Montpellier, France)
Rémi Charrel (University of Marseilles, France)
Marie Dewannieux (University College, London, UK)
Jean-Michel Drezen (University of Tours, France)
Santiago Elena (IBMCP, Valencia, Spain)
Rémy Froissart (CNRS, Montpellier, France)
Barbara Hohn (Friedrich Miescher Institute, Basel, Switzerland)
Thomas Hohn (Basel University, Switzerland)
Mireille Jacquemond (INRA Montfavet, France)
Edgar Maiss (U Hannover, Hannover, Germany)
Marcel Prins (Keygene, Wageningen, The Netherlands)
Katja Richert-Pöggeler (IPK, Gatersleben, Germany)
Ilan Sela (Hebrew University of Jerusalem, Israël)
Trude Schwarzacher (University of Leicester, UK)
Christina Staginnus (Gregor Mendel Institut, Vienna, Austria)
Mark Tepfer (ICGEB, Ca'Tron, Italy)
Pierre-Yves Teycheney (CIRAD Guadeloupe, FWI)

Organizing committee

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The first BIOSAFENET Seminar

Balancing resistance and risk: plant endogenous viral sequences and virus-resistant transgenic plants as possible sources of resistance and virus emergence

6-8 June 2007

International Centre for Genetic Engineering and Biosafety (ICGEB)
Ca' Tron di Roncade, Italy

Wednesday 6th June 2007

5:30 pm – 6:00 pm : Presentation of the participants and scientific programme
6:00 pm – 7:30 pm : Refreshments and presentation of the Biosafenet project, ICGEB & Fondazione Cassamarca

- **Joachim Schiemann (BBA, Braunschweig, Germany; coordinator of the Biosafenet project)**
- **Antonio Zamboni (Fondazione Cassamarca, Ca'Tron di Roncade, Italy)**
- **Decio M. Ripandelli (ICGEB, Trieste, Italy)**

7:30 pm – 9:00 pm : Dinner

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Thursday 7th June 2006

8:30 – 8:45 : Setting the stage on VRTPs
- **M. Tepfer (ICGEB, Italy)**

8:45 - 10:15 - Session 1 : VRTPs and virus resistance mechanisms

- **Marcel Prins (Keygene, The Netherlands):** Resistance strategies available against Tospoviruses
- **Thomas Hohn (University of Basel, Switzerland):** Plant viruses and siRNA

10:15 – 10:30 - Coffee Break

10:30 – 12:30 – Session 2 : Biosafety of VRTPs

- **Mark Tepfer (ICGEB, Ca'Tron, Italy) :** Evaluation of potential risks associated with recombination in virus-resistant transgenic plants expressing viral sequences
- **Mireille Jacquemond (INRA Montfavet, France) :** Biological properties and relative fitness of inter-subgroup *Cucumber mosaic virus* (CMV) RNA 3 recombinants
- **Edgar Maiss (U Hannover, Hannover, Germany) :** No recombination detected in artificial potyvirus mixed infections and between potyvirus derived transgenes and heterologous challenging potyviruses

12:30 – 14:00 Lunch

14:00 – 16:00 – Session 3 : Virus evolution and emerging viral diseases

• **Rémy Froissart (CNRS Montpellier, France) :** Adaptation of a phytovirus to its environment : evolution of the genome linked to the evolution of virulence
• **Santiago Elena (IBMCP, Valencia, Spain) :** The evolution of silencing suppression and other ways viruses have to escape from silencing
• **Barbara Hohn (FMI, Basel, Switzerland) :** Transgenerational memory of stress in plants

16:00 – 16:15 – Coffee break

16:15 – 17:45 – Discussion

18:00 – 19:00 – Visit of ICGEB Biosafety outstation & refreshments

19:00 – 20:30 – Dinner

20:30 – 21:30 : Wine testing at the Castello di Roncade

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Friday 8th June 2006

8:30 – 8:45 : Setting the stage on EPRVs
• **Pierre-Yves Teycheney (CIRAD, France)**

8:45 – 12:00 - Session 4 : Endogenous viral sequences in the genome of plants

• **Trude Schwarzacher (University of Leicester, UK) :** Organisation and relationship of LTR-retroelements and EPRV-like sequences in plant species including banana and sugar beet
• **Chisitna Staginnus (Gregor Mendel Institut, Vienna, Austria):** Endogenous pararetroviral sequences (EPRVs) in tomato (*Solanum lycopersicum*) – a tamed class of repetitive elements?
• **Katja Richert-Pöggeler (IPK, Gatersleben, Germany):** Inducible endogenous *Petunia vein clearing (pararetro) virus*: communication, competition and control in a viral *ménage à trois* (ePVCV, petunia and PVCV)

10:45 – 11:00 : Coffee break

11:00 – 12:30 – Session 4 (cont'd)

• **Marie-Line Caruana (CIRAD, Montpellier, France):** The exploration of the pathosystem BSV/*Musa* sp.: How does it work?
• **Ilan Sela (Hebrew University of Jerusalem, Israël) :** Reciprocal exchange of genomic information between viruses and their hosts

12:30 – 13:30 – Lunch

13:30 – 15:30 - Session 5 : Endogenous viral sequences in the genome of mammals and insects

• **Jean-Michel Drezen (University of Tours, France):** Virulence genes of parasitoid wasps encoded by symbiotic viruses
• **Rémi Charrel (University of Marseilles, France) :** DNA forms of non-retroviral animal RNA virus genomes in eukaryotic cells
• **Marie Dewannieux (University College, London, UK) :** Mechanisms of the mobility of mammalian endogenous retroviruses

15:30 – 16:00 – Roundtable : Reconsidering the biosafety of VRTPs in the light of EPRVs and vice versa.

Should the results concerning EPRVs modify our conception of the biosafety of virus-resistant transgenic plants?

16:00 – 16:30 – Concluding remarks and future collaborations

19:00 – 21:30 : Refreshments & conference dinner

Resistance strategies available against Tospoviruses

Marcel Prins

Pathogen resistance has been an important trait in the breeding of crop plants. Although natural sources of resistance have proven highly valuable against some species of virus, their use has been limited for others. Over the last two decades or so biotechnology has produced additional tools to combat the highly divergent viral pathogens, some enhancing existing antiviral strategies in plants, others presenting completely novel concepts. In this presentation an overview of examples produced over the years for the important group of Tospoviruses will be outlined.

Tomato spotted wilt virus is the type member of the negative strand RNA *Tospovirus* genus and economically the most relevant virus of this group, although other members of this genus have been implicated in outbreaks of these viruses in vegetable and ornamental plant production world wide. The list of recognized members of this genus has continued to grow over the last decade or so and currently includes over 15 species. Many tospoviruses have wide host ranges including many crops as well as weed species that can serve as a reservoir for the virus. All tospoviruses are transmitted by one or more species of thrips, insects that have demonstrated a great capacity to rapidly develop resistance against insecticides.

Limited natural sources of resistance against tospoviruses are available, but durability in the field often proved to be insufficient. To prevent dramatic losses in crop yield, alternative methods for resistance against tospoviruses were explored. This presentation summarizes several of the currently available options for introducing or transferring natural and artificial forms of resistance to a range of crop plants affected by tospoviruses. These include the characterization and cross-species application of the tomato gene Sw5 -which confers a broad spectrum of resistance to tospoviruses-; the application of transgenic expression of antibody fragments in transgenic plants and the pre-programming of the plant's RNA silencing defense mechanism by producing virus specific siRNAs. Potential for use in the field, including risks for recombination between transgenic and viral RNA molecules and durability of resistance strategies will be discussed.

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Plant viruses and siRNA

Thomas Hohn [1], Mikhail M. Pooggin [1], Rashid Akbergenov [1], Padubidri V. Shivaprassad [1], Faiza Noreen [1], Katja Richert Pöggeler [1, 4] Todd Blevins [2], Frederick Meins [2], Karuppannan Veluthambi [3] & V. Balamani [3]

A virus infection establishes a battlefield involving virus attack, host defense and viral counter-defense. The outcome of the battle can range from total resistance to severe infection. In plants, one of the main defense strategies is RNA interference (RNAi). It involves recognition of the invading nucleic acid and its destruction as well as the systemic warning of the whole organism. The main target of silencing is double-stranded RNA (dsRNA) formed by viruses as a byproduct of transcription or replication. dsRNA is cleaved by dicers into small interfering RNAs (siRNAs), which become incorporated into a RISC complex to act as guides to cleave cognate RNA molecules. Resulting fragments can be used as templates to produce more dsRNA. Upon infection of plants with viruses that are established in the nucleus as minichromosomes, i.e. caulimoviruses and geminiviruses, or of induction of endogenous plant pararetroviruses integrated as fragments into the host chromatin, their transcripts are attacked by all of the four available dicers. In contrast, RNA viruses are only attacked by two of them, DCL4 and DCL2. This shows that virus silencing is established in whatever compartment is affected by the infection and that elements of both, transcriptional silencing (TGS) and posttranscriptional silencing (PTGS) are involved.

Some of the virus encoded proteins, the suppressors, interfere with silencing. Most commonly, viral suppressors block siRNA usage by binding to it. However, in begomo-geminiviruses, the suppressor is a viral transcription factor inducing several host genes, at least one of them controls silencing and the *Cauliflower mosaic virus* transctivator/viroplasmin interferes with processing of ds RNA precursors of viral and transcting siRNAs.

Hosts react to plant viruses in a range from total resistance to severe infection. However, even in severe infections, recovery can occur and in most viral infections the meristem and some new growth is spared. This phenomenon can be visualized by virus induced gene silencing (VIGS) using Arabidopsis and *Cabbage leaf curl begomovirus* carrying sequences of the host chlorata gene. In this case, VIGS is seen as bleaching of the new growth area. Moreover, the new growth is most active in transitivity, i.e., the production of siRNAs from RNA not covered by the viral payload but present downstream of the cognate region of the targeted host RNA. Transitivity is indicative of amplification of dsRNA and depends on RNA-dependant RNA polymerase 6 (RDR6) and DCL1. Consequently and in contrast to the primary siRNAs, the secondary siRNAs produced through transitivity are predominantly of the 21 nt size class.

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Evaluation of potential risks associated with recombination in virus-resistant transgenic plants expressing viral sequences

Camilla Turturo [1], Arianna Friscina [1], Stéphane Gaubert [2], Mireille Jacquemond [3], Jeremy R. Thompson [1] & Mark Tepfer [1, 2]

Virus-resistant transgenic plants (VRTPs) have been created primarily through the expression of viral sequences. It has been hypothesized that recombination between the viral transgene mRNA and the RNA of an infecting virus could generate novel viruses. Since messenger/viral RNA recombination can occur in VRTPs, the key to testing this risk hypothesis is to compare the populations of recombinant viruses generated in transgenic and non-transgenic plants under conditions of low selection pressure in favor of the recombinants. We have done this with two cucumoviral systems, involving either two strains of *Cucumber mosaic virus* (CMV) or CMV and the related *Tomato aspermy virus* (TAV). Although the distribution of the sites of recombination in the CMV/CMV and TAV/CMV systems was quite different, in both cases equivalent populations of recombinant viruses were observed in transgenic and non-transgenic plants. This suggests that there is little risk of emergence of recombinant viruses in these VRTPs, other than those that could emerge in non-transgenic plants. In addition, these results suggest that inter-viral recombination may create a cloud of variants that would be an integral part of the complexity of populations of viral RNA molecules.

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Biological properties and relative fitness of inter-subgroup *Cucumber mosaic virus* (CMV) RNA 3 recombinants

Olivier Pierrugues [1], Laurent Guilbaud [1], Isabelle Fernandez-Delmond [2], Frédéric Fabre [1], Mark Tepfer [2, 3] & Mireille Jacquemond [1]

Co-infection of tobacco plants with CMV-I17F and R strains, representative of subgroups IA and II, respectively, results in viral RNA populations including rare recombinant RNA 3 molecules, some of which also have point mutations. A small set of recombinants, representing a typical sampling of the total sequence variability produced by mutation and recombination, was selected for assessing the possibility for these molecules to be positively selected in infected plants. The biological properties of 17 recombinants in the capsid gene or the 3' non-coding region of RNA 3 were evaluated when associated with I17F RNAs 1 and 2. Six viruses displayed important deficiencies (non-viability, deficiencies for movement and/or replication, irregular or delayed infection, loss of aphid-transmissibility). Among the 11 perfectly viable recombinants, nine evoked symptoms close to those of I17F-CMV on tobacco and pepper plants. In addition, all recombinants bearing the movement protein of R-CMV and part or most of the capsid protein of I17F-CMV, as well as the recombinant created *in vitro* by exchanging the corresponding open reading frames, induced severe filiformism on tobacco. They however evoked only faint symptoms on melon, suggesting that they would be strongly counter-selected in this host if they indeed occurred in the field. The two remaining recombinants evoked atypically severe symptoms on both tobacco and pepper. Most of the recombinants generally accumulated to lower levels in tobacco; only five had levels of viral RNA accumulation that were equivalent to the wild-type and thus could be considered as potentially competitive. Among them, three recombinants however, including one responsible for severe symptoms, accumulated to levels generally higher than I17F-CMV. When two of these were tested in co-infection experiments with I17F RNA 3, they proved to be poorly competitive. Although it is not possible to formally exclude that a given recombinant might be positively selected in a host species other than the three studied in this work, considering the breadth of the CMV host range, our results suggest that the possibility for CMV IA/II recombinants to emerge would be rare in the field. This is consistent with the fact that first, plants expressing the atypical severe symptoms have never been described in nature and second, that natural CMV recombinant isolates remain rare. Moreover, since equivalent populations of recombinants are observed in virus-resistant transgenic plants expressing a CMV-capsid sequence and co-infected non-transgenic ones, our results also suggest that there is little risk of emergence of recombinant viruses in transgenic plants.

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No recombination detected in artificial potyvirus mixed infections and between potyvirus derived transgenes and heterologous challenging potyviruses

Christof Dietrich [1], Jane Miller [2], Gaynor McKenzie [2], László Palkovics [3], Ervin Balázs [4], Peter Palukaitis [2] & Edgar Maiss [5]

The use of virus-resistant transgenic plants (VRTPs) in agricultural and horticultural crop production systems offers a considerably effective strategy to protect plants from severe diseases caused by viruses. Since the first report of a VRTP in 1986 numerous plants have been developed that confer resistance to plant viruses. However, beside the benefits of VRTPs, it has been suggested that the release of transgenic plants into agroecosystems might engender ecological risks (reviewed by Tepfer, 2002). Mainly two types of potential risks arising from VRTPs have to be taken into account: firstly, recombination, secondly heterologous encapsidation, complementation and synergy. Whereas the latter cases could lead to temporary phenotypic effects, recombination will permanently affect the genetic makeup of a viral genome and thus, has been assumed to be of greater impact.

To evaluate possible risks associated with VRTPs risk-assessment studies focussing on recombination of a plant virus with a transgenic sequence of a different virus should be carried out. This kind of study should also include a comparison of recombination events between viruses in doubly infected non-transgenic plants with those observed in singly infected transgenic plants to estimate recombination incidences in both situations.
Potyviruses, like potato virus Y (PVY), plum pox virus (PPV) or tobacco vein mottling virus (TVMV) cause severe agronomic losses. Here, the occurrence of recombination events was investigated in non-transgenic plants doubly infected with different potyvirus combinations, as well as in singly infected transgenic plants expressing heterologous potyvirus sequences. Different potyviruses, namely potato virus A (PVA), the PVY strains O and H, two strains of PPV (NAT, SK68) and TVMV were used in three combinations for double infection of a common host. Furthermore, transgenic plants expressing either potyviral coat protein (CP), helicase (CI) or polymerase (NIb) coding sequences (PPV-NAT-CP, PVY-CI, PVY-NIb) were singly infected with a heterologous potyvirus, which was not targeted by the respective transgenic resistance. To identify recombinant potyviral sequences, a sensitive RT-PCR was developed, which is able to detect up to one recombinant molecule out of 10^6 parental molecules. In over 300 mixed infected non-transgenic plants, over 90 mixed and over 160 singly infected transgenic plants screened for recombinant sequences no recombinant potyviral sequence was detected. The possible reasons for the failure to detect recombinant potyviral genomes will be discussed.

Tepfer M.2002. *Annu Rev Phytopathol*. **40**:467-91

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Adaptation of a phytovirus to its environment :
evolution of the genome linked to the evolution of virulence

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Phenotypic expression of mutations is of particular interest to our understanding of life processes, especially when it concerns the evolution of pathogens. The study of phenotypic expression is important because natural selection acts on phenotypic expression of mutations in both constant and variable environments. In viruses, phenotypic expression of mutations may be manifested not only in symptoms but also in the levels of viral fitness (reproductive capacity of a genotype) and virulence (decrease in host fitness).

We have proposed to follow the experimental evolution of *Cauliflower mosaic virus* (CaMV, Caulimovirus). CaMV is an appropriate biological model because we have a good knowledge of its biological cycle and also because it presents a high evolution rate (high mutant frequencies and recombination rate) and a large host range. Viral populations (minimum of 10 lineages per treatment) have been transmitted from plant to plant in either two homogeneous environments (*Arabidopsis thaliana* or *Nicotiana bigelovii*) or a variable environment (alternating both species). After 5 passages (approx. 35 to 50 generations), we determined the full genome length consensus sequence of viral lineages, and looked for parallel mutations that similarly appeared in independent populations that were subjected to similar directional selective pressures, such as adaptation to the environment. Because CaMV is easy to manipulate (e.g. cloning, sequencing, etc), we will readily determine the kinetics of accumulation of adaptive mutations, and test their phenotypic expression when alone or in combination in all types of environments (i.e. host plant genotypes).

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The evolution of silencing suppression and other ways viruses have to escape from silencing

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It is well established now that viruses are both initiators and targets of RNA silencing, a mechanism used by plants as an antiviral adaptive response (Baulcombe 2004; Lecellier & Voinnet 2004; Waterhouse *et al*. 2001). However, viruses have evolved counterdefenses that allow them to escape from silencing surveillance and successfully infect the plant. Such diverse mechanisms range from the high genetic variability and adaptability bestow by the quasispecies nature of viral populations to the acquisition of suppressor activities, usually encoded by multifunctional proteins (Kasschau & Carrington 1998; Qu & Morris 2005; Voinnet *et al*. 1999). In the laboratory, we are now applying evolutionary analyses to understand the implications of these two mechanisms on the evolution of two potyviruses (TEV and TuMV). I will present some preliminary results from these two studies.

In a first set of experiments, we have created a collection of 27 amino-acid replacement mutants in TEV's silencing suppressor, HC-Pro, and evaluated their efficiency as suppressors in transient-coexpression assays with GFP in *Nicotiana benthamiana* plants. In parallel, for each mutant protein we have evaluated the ability of the virus to move systemically, their accumulation and the severity of the symptoms induced. Nine mutants showed no activity at all, three showed significant reduction in suppressor activity (hyposuppressors), nine mutations were neutral, and five were stronger suppressors than wildtype (hypersuppressors). All hyposuppressors accumulated less and induced milder symptoms than wildtype TEV. Hypersuppressor were most variable in their accumulation and symptomatology. Now, we are running serial transfer evolution experiments with three mutants from each category. The goal is to explore whether different lineages would convergence to the phenotypic value of the wildtype virus (stabilizing selection) or different degrees of suppression would be evolutionarily stable (disruptive selection).

It has been recently proposed that expression of amiRNAs targeting TuMV HC-Pro may be an efficient way of generating resistant plants (Niu *et al*. 2006). However, HIV-1 populations evolved on cells that stably expressed siRNAs specifically designed to target viral proteins rapidly generated escape mutants (Boden *et al*. 2003; Das *et al.* 2004; Westerhout *et al*. 2005) by evolving alternative sequences that affected the target. These observations prompt caution against the use of amiRNA-derived resistances. In cooperation with Prof. N.H. Chua, we have started a large-scale evolution experiment in which TuMV populations are serially transferred on *Arabidopsis thaliana* Col-0 and, after each transfer, evaluated for their ability to replicate on the amiRNA-HC-Pro transgenic plants. This experiment simulates the generation of neutral or quasi-neutral genetic variability in viral populations replicating on a wildtype host that periodically are challenged to replicate on the resistant plants.

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Transgenerational memory of stress in plants

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Plants are constantly exposed to stresses from the environment. Both biotic and abiotic stresses can result in tolerance to conditions such as unbalanced water supply, light stress, extreme temperatures and pathogens. Influences such as these have also been documented to result in elevated rates of genomic changes including transposition and homologous recombination. However, it has not been analysed whether the impact of these influences could also be inherited. Experiments using *Arabidopsis thaliana* plants transgenic for markers for genomic changes showed that exposure of these plants to UV-B or to the bacterial elicitor flagellin exhibited changes of homologous recombination far exceeding those of unexposed plants. Quite unexpectedly, however, the progeny of these plants still had elevated recombination rates, although they themselves were not influenced by the agents their parents were exposed to. This change in the recombination frequency must be due to an epigenetic modification of an unknown locus or of unknown loci; it was the whole population that reacted to the challenge. If a mutation would have caused the described affect only a very small number of plants would have shown the changes behavior. The change was not located at the genomic position of the transgene used to monitor recombination, since plants lacking the transgene transmitted their experience of being treated to an untreated crossing partner containing the recombination monitoring transgene. This resulted in increased recombination frequencies in the progeny of these plants. These experiments may point to the importance of epigenetic changes of plant chromatin in permitting evolutionarily important flexibilities useful for adaptation.

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Organisation and relationship of LTR-retroelements and EPRV-like sequences in plant species including banana and sugar beet

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Several evidences have shown that retroviruses are closely related to some long terminal repeat (LTR) retrotransposons (Xiong and Eickbush, 1990). We have used extensive bioinformatic comparisons of DNA and amino acid sequences and found that the RT domain of pararetroviruses shows indeed many similarities to that of *Metaviridae* retroelements, while the RNAse H domain contains several significant motifs that clearly separate pararetroviruses from *Metaviridae* and puts them into seven significant clades. Molecular cytogenetic analysis have shown that endogenous pararetroviruses (EPRVs) are located at paracentromeric positions that are rich in retroelements indicating a further relationship between the two elements with possibly linked amplification mechanisms. In *Musa* genomes, we studied LTR- retroelements extensively and identified three different groups of *Metaviridae* that show different levels of degeneracy and postulated activity. We were also interested to confirm the indications for a wealth of additional EPRV sequences in the whole plant world. While the amino acid sequence of EPRVs is highly conserved, at the DNA sequence level much greater degeneracy is found, such that a universal PCR approach to isolate further EPRVs has been difficult. However, using a double PCR approach with nested and semi-nested primers, we have been able to isolate EPRV sequences from diverse plant species including Norway spruce, pine, brassica, olive, barley and sugar beet. In sugar beet, we have shown that a few hundred EPRVs are present in the genome and that they are clustered on selected BAC sequences.

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Endogenous pararetroviral sequences (EPRVs) in tomato (*Solanum lycopersicum*) a tamed class of repetitive elements?

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Endogenous pararetroviral sequences (EPRVs) represent a recently discovered class of repetitive sequences that is broadly distributed in the plant kingdom. EPRVs have been characterized in various species of the family Solanaceae including *Nicotiana tabacum* (tobacco) and *Solanum tuberosum* (potato). Endogenous *Tobacco vein clearing virus* (TVCV) in *Nicotiana edwardsonii* probably gives rise to episomal, infectious particles, but no horizontal transmission has been reported. Further EPRVs in the genomes of *Nicotiana* and *Solanum* species are similar to TVCV in sequence and structure and form a TVCV-like group.

Recently, a TVCV-related family of EPRVs named *Lyc*EPRVs has been characterized in cultivated tomato (*Solanum lycopersicum* L.) and a wild relative (*S. habrochaites).* The sequence similarity in *S. lycopersicum* and *S. habrochaites* indicates that they are potentially derived from the same pararetrovirus. A similar genomic organization in the two species was revealed by DNA blot analysis, but also some independent excision or insertion events after species separation or flanking sequence divergence can be detected. Similar to tobacco elements, *Lyc*EPRVs show a frequent association with retrotransposons and a disrupted genomic structure. Fluorescence *in situ* hybridization revealed that copies of *Lyc*EPRV are dispersed on all chromosomes in predominantly heterochromatic regions.

Methylation of *Lyc*EPRVs was detected in CHG and asymmetric CHH nucleotide groups. A reactivation or symptoms of infection caused by *Lyc*EPRVs could not be demonstrated in *Solanum* L. section *Lycopersicon* [Mill.] hybrids. However, transcripts derived from multiple *Lyc*EPRV loci and short RNAs complementary to *Lyc*EPRVs were detected even in healthy plants and were elevated upon infection with heterologous viruses encoding suppressors of PTGS. The detection of asymmetric CHH methylation and short RNAs, which are hallmarks of RNAi in plants, suggests that *Lyc*EPRVs are controlled by an RNA-mediated silencing mechanism.

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Inducible endogenous Petunia vein clearing (pararetro) virus: communication, competition and control in a viral *ménage à trois* (ePVCV, petunia and PVCV)

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Endogenous pararetroviruses have by definition abandoned their episomal stage and became manifested in their host genome. Accordingly horizontal virus transmission from plant to plant has been replaced by vertical transfer of virus sequences via reproduction cells. Evidence accumulated so far, indicates that integration does not occur actively as known for some bacterial or animal viruses but as a “by-product” of the DNA repair machinery of the host.

We identified integrated *Petunia vein clearing virus* (ePVCV) sequences in *Petunia hybrida* cultivars Rose du ciel (RdC) and W138. The selected hybrids are thought to be genetically not uniform but show a similar copy number and distribution of the investigated retroelement. Some integrants within the petunia genome are arranged in tandem, allowing direct release of virus by transcription. Indeed, ePVCV can be activated in cultivars RdC and W138 to trigger a systemic virus infection. However, cultivar W138 showed a higher sensibility to inducing conditions like wounding or abiotic stress indicating less efficient ePVCV-control than cultivar RdC. Endogenous PVCV sequences were located in the pericentromeric region of petunia chromosomes comprising heterochromatin that in general is transcriptionally inactive. Despite the positioning in heterochromatin we could observe continuous transcription of ePVCV sequences. This is in good agreement with the hypothesis that initial transcription is required to prime various RNAi pathways and provided a first indication that gene silencing may be involved in their transcriptional control. To further resolve the mechanisms underlying possible epigenetic regulation, we looked at chromatin methylation in the two hybrid petunia lines. We found that DNA hypermethylation and histone methylation is associated with silencing of the endogenous copies of PVCV in the two cultivars of *P. hybrida*. Unexpectedly, only an extremely low amount of ePVCV-derived siRNAs was found in line W138 when uninduced. In the non-induced line RdC, ePVCV-specific siRNAs could not be observed at all. The scarcity of siRNAs might in fact be an indication for very successful silencing, because of the lack of transcripts that are the source of siRNAs.

While DNA methylation patterns were the same in both lines, histone methylation patterns clearly differed in Rdc and W138. The predominant association of ePVCV sequences with histone H3 methylated at lysine 9 (H3mK9) in RdC and with about equal amounts of H3mK9 and H3mK4 in W138 indicates a less repressive proviral state in the latter cultivar.

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The exploration of the pathosystem BSV/*Musa* sp.: How does it work?

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As several other plants, the genome of banana and plantain contains integrations of *Banana streak virus* (BSV) sequences even though integration is not an essential step in the replication cycle of this virus. In banana two types of BSV integrants exist. Ones are non functional sequences present in both common *Musa* species, *Musa acuminata* (denoted A) and *Musa balbisiana* (denoted B) and it is now assumed that the integrants of the other type, containing the complete viral genome and restricted to *M. balbisiana* genome, become infectious by reconstituting a complete replication-competent viral genome. Thereby, an increasing record of BSV outbreaks was observed fifteen years ago among banana breeding lines and micro propagated inter-specific *Musa* hybrids, worldwide.

Today, three widespread BSV species, *Banana streak Obino l'Ewai virus* (BSOlV), *Banana streak Imové virus* (BSImV) and *Banana streak Golfinger virus* (BSGfV) are known to occur as infectious integrants in the *M. balbisiana* genome. However, even though such integrations are known to be infectious, their presence is not sufficient to induce infection

We demonstrated that the process of genetic hybridization and abiotic stresses such as micropropagation by *in vitro* culture contributed in triggering episomal expression from EPRVs. Two mechanisms at least are involved in the BSV expression: the ploidy of the *M. balbisiana* in *Musa* genotypes and an additional genetic factor called BEL for BSV expressed locus concerning the triploids (*Musa* AAB) resulting from inter-species genetic crosses between virus-free diploid *M. balbisiana* (BB) and tetraploid *M acuminata* (AAAA) parents. Then, diploids *M. balbisiana* such as PKW and Pisang Batu harboring pathogenic BSV EPRVs are resistant to any multiplication of BSV while haploid genotypes such as triploids (AAB, French clair) or tetraploids (AAAB, FHIA 21) expressed BSV. Thereby, we characterized the segregation of three BSV species appearance among the AAB F1 progeny as a monogenic allelic system conferring the role of carrier to the *M. balbisiana* diploid parent. BSOlV and BSImV appeared in almost all infected hybrids (50% of the progeny) depending of BEL regulation while BSGfV are restricted in only half of these hybrids and subordinated by BEL.

Three BAC libraries from accession of *M.acuminata* Cavendish subgroup cv petite naine (AAA), a wild *M. acuminata* subsp *burmannicoides* Calcutta 4 (AA) and a wild *M. balbisiana* PKW (BB) are explored for the pattern of integration of infectious BSV EPRVs by testing a set of different viral probes representing each time the BSOlV, Im and Gf complete genome.

BSV positive BAC clones are characterised by RFLP fingerprints approaches. This analysis revealed that the three BSV species represent low-copy loci and their integration is specific to the PKW *Musa balbisiana* genome. BSGfV EPRVs in PKW is twice and are fully annotated after sequencing. Each BSGfV is composed of back-to-back viral sequences representing more than a whole BSV genome very similar each other. We developed molecular markers (PCR, PCR-RFLP) to distinguish each others and analysed the BSGfV EPRV segregation in the AAB F1 progeny. BSGfV EPRVs are found to be allelic, located at the same locus. In theory, both allelic EPRVs could be involved in the restitution of virions through a set of recombination events.

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Reciprocal exchange of genomic information between viruses and their hosts

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Sequences of non-retro-viruses have been found in plant and insect genomes, and reciprocally, host sequences have been found fused to viral-derived defective RNAs. All such chimeric host-virus sequences were flanked by direct and/or inverted repeats, also found within the sequences themselves. Analysis of these sequences suggested that the process (or processes) leading to virus:host sequence fusion involve RNA recombination, followed by reverse transcription, integration into the genome and expression therefrom. However, virus-aided transmission also appears to occur, such that the chimeric constructs may be horizontally transferred from host to host by chimera-carrying virions (Tanne and Sela, *Virology* 332: 614-622, 2005; Maori et al., *Virology* 362: 342-349, 2007). In addition, *in-silico* genome and EST searches have yielded a multitude of virus:host chimeric sequences, indicating that such fusions, as infrequent as they may be, are not negligible.

We hypothesize that RNA recombination can take place between any two RNAs in the absence of virus. However, the phenomenon is virus-stimulated because viral RNAs are abundant, and the virus provides RNA-dependent RNA polymerase which is capable of template switching as well as of stimulating the activity of retrotransposons, leading to the formation of DNA versions of viral RNAs and their subsequent integration into host genomes. There are indications that the genome-integrated sequences are unstable and undergo further DNA recombination, leading to their deterioration within a few generations. However, if integrated within an essential gene without abrogating its function, the viral sequence is preserved and expressed. The inserted foreign sequences are also preserved in asexually reproducing organisms, as recombination takes place only during meiosis.

The described phenomenon suggests a way in which divergence within individuals develops. The various hypothetical pathways leading to the aforementioned sequence fusions are currently being studied experimentally and will be presented.

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Virulence genes of parasitoid wasps encoded by symbiotic viruses

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Hundreds of thousand species of wasps develop during their larval stage within the body of other insects while the adults are free. In particular 17500 species of wasps, phylogeneticaly related -the microgastoids- are obligatorily associated with viruses of the bracovirus genus. Permanently integrated into the wasp chromosomes, bracoviruses are thought to originate from the same virus integrated in the genome of the ancestor of the microgastroids that lived approx. 70 millions years ago. Bracoviruses are essential for parasitism success. Mature virions are produced in the wasp ovaries and injected by female into the host lepidopteran larvae, along with the wasp's eggs. The virus particles enter host cells where viral genes are expressed, causing several alterations to the host physiology which are beneficial for the development of the wasp progeny, comprising disruption of the immune defenses, retarded growth and inhibition of metamorphosis

Sequencing of a bracovirus genome have allowed the characterization of several gene families encoding potential factors involved in parasitism success. Interestingly some of the genes recently identified contain conserved protein domains found in virulence factors from other parasites or pathogens that are known to be involved in the suppression of the immune response of mammals. The characterization of genes conserved in the virus genomes of different parasitoid species will also be an essential tool to understand the role played by bracoviruses in the radiation of the parasitoid wasp families, highly diversified.

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DNA forms of non-retroviral animal RNA virus genomes in eukaryotic cells

Rémi Charrel & Xavier de Lamballerie

The presence of DNA forms of non-retroviral animal RNA virus genomes has been reported repeatedly in eukaryotic cells. Unfortunately, these data are sparse, scattered and rarely reproduced by experimental data. A brilliant exception is represented by the infection of mice by the lymphocytic choriomeningitis virus. We present here a historical summary of scientific data that support the existence of biological mechanisms allowing the synthesis of DNA forms of non-retroviral animal RNA virus genomes in eukaryotic cells. In addition, Flavivirus-related sequences have been discovered in the dsDNA genome of *Aedes albopictus* and *Aedes aegypti* mosquitoes, demonstrating for the first time integration into a eukaryotic genome of a multigenic sequence from an RNA virus that replicates without a recognized DNA intermediate. In the *Aedes albopictus* C6/36 cell line, an open reading frame (ORF) of 1557 aa with protease/helicase and polyprotein processing domains characteristic of flaviviruses was identified. It is closely related to NS1-NS4A genes of the Cell Fusing Agent and Kamiti River virus and the corresponding mRNAs were detected. Integrated sequences homologous to the envelope, NS4B and polymerase genes of flaviviruses were identified. Overall, approximately two-thirds of a flavivirus-like genome was characterized. In the *Aedes aegypti* A20 cell line, a 492 aa ORF related to the polymerase of the Cell Fusing Agent and Kamiti River virus was identified. These flavivirus-related integrated DNA sequences were detected in laboratory-bred and wild *Aedes albopictus* and *Aedes aegypti* mosquitoes, demonstrating that their discovery is not an artefact resulting from the manipulation of mosquito cell lines, since they exist under natural conditions. Importantly, the in-cellulo synthesis of DNA forms of viral genomes could be obtained experimentally following the infection of mosquito cells by CFA and KRV. The phenomenon proved to be resistant to the action of common anti-retroviral molecules. Altogether, this finding has major implications regarding evolution, as it represents an entirely different mechanism by which genetic diversity may be generated in eukaryotic cells distinct from accepted processes. Recent analysis of databases showed identified RNA virus sequences integrated in various eukaryotic genomes.

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Mechanisms of the mobility of mammalian endogenous retroviruses

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Mammalian Endogenous Retroviruses are thought to be the remnants of ancestral infections by active retroviruses which have thereafter been transmitted in a Mendelian manner and are still closely related to their infectious counterparts. The endogeneization process has been accompanied by a co-evolution of their new host, enabling it to regulate these elements, both by controlling their expression and by directly interfering with their replicative cycle via protein restriction factors. In the course of evolution, parts of these elements have also been co-opted and are now used for the protection of the organism against modern retroviruses and for other physiological functions, as exemplified by the involvement of the *syncytin* genes in the formation of the placenta. Independently of these roles, some endogenous retrovirus families are still active on their own and can increase their copy number within the genome through their replicative cycle. In the case of the human HERV-K family, we derived *in silico* the sequence of its putative ancestral "progenitor" element and constructed an expression vector for it. This element produces viral particles which disclose all the structural and functional properties of a *bona fide* retrovirus, can infect mammalian -including human- cells and integrate with the exact signature of the presently found endogenous HERV-K progeny. We also showed that this element amplifies via an extra cellular pathway, involving re-infection of neighboring cells, thus recapitulating *ex vivo* the molecular events responsible for its dissemination in primate genomes. The study of other families of endogenous retroviruses isolated from the murine genome shows that other mechanisms of amplification can be effective: we demonstrated that the IAP and MusD elements can replicate within mammalian cells by a strictly intracellular process at a very high rate. The functional elements we characterized encode unusual retroviral-like particles, assembled and stored within the cytoplasm, which probably accounts for the efficiency of the amplification process since these particles can not be diluted in the extracellular compartment. In both cases the relocalization of the particles inside the cells is performed by a modification of the addressing of the Gag protein and a loss of the envelope protein (required for the extra cellular steps of the retroviral life cycle).

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